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Molecular-Biological Marker for Analytical Electron
Microscopy

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BACKGROUND OF THE INVENTION

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The invention relates to a series of new plasmids on the basis of pBluescript KS(+), comprising more than 1 SK primer sequence element, preferably 2, 7, 14, 21 and 27 repetitive SK primer sequence elements, and the use thereof as a molecular-biological marker for analytical electron microscopy.

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Electron spectroscopic imaging (ESI) is a method of analytical electron microscopy (EM), which pictures the distribution of a certain chemical element within the analyzed preparation. In order to elucidate the structural organizations of biological systems, it must be possible to optically differentiate the individual macromolecular components. At present, the charging with gold particles or other particles, which are visible in the refraction contrast, is used to label macromolecules for electron microscopy.

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Thus far, multiple labeling experiments have been carried out in electron microscopy by using gold grains of various sizes to be able to differentiate the different target structures in a single preparation. For example, one molecule type would be linked to gold grains having a size of 5 nm while the other would be attached to those having a size of 10-20 nm in a double labeling experiment to ensure that in a subsequent evaluation the different molecules can clearly be localized and distinguished from one another.

Large gold grains (larger than 10 nm) are disadvantageous because they have reduced penetration capacity into the tissue and reduced coupling efficiency to the target molecule (Giberson, T.R., and Demaree, R.S.: The influence of immunogold particle size on labeling density. Microscopy Research and Technique, 27, 355-357, 1994). In addition, such a large structure can no longer be assigned clearly to the site of binding to the target structure, i.e. resolution capability is lost. If a triple labeling experiment was aimed at, these drawbacks would become particularly striking. Only what is called ferritin molecules, i.e. large protein units which contain hundreds of iron atoms in their centers and can be linked to target structures, are an alternative to the gold grains. However, their electron density and thus their detectability under the transmission electron microscope is very poor so that their use has only proved feasible in rare cases.

In contrast thereto, florescence methods which enable triple labeling, and even quadruple labeling, without causing major problems have existed in the field of optical microscopy for some time now. Since as regards the labeling techniques electron microscopy cannot compete with optical microscopy for the time being, scientists are satisfied with the comparatively poor resolution capability of optical microscopes before they accept the drawbacks accompanying the labeling technology in the electron-microscopic field. The development of alternative labeling techniques for gold labeling would render electron microscopy more attractive because its competitiveness as regards labeling would be accompanied by a resolution capability over 100 times as good as that of optical microscopy. As an alternative to the labeling method using gold for the conventional transmission electron microscopy, which is based on the electron density

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of the heavy metal gold, there is a demand for labeling methods for ESI. This technique utilizes interactions between beam electrons and the atoms in the preparation differing from those of conventional transmission electron microscopy. In principle, all of the elements can be detected specifically. This raises the number of elements in consideration for labeling methods. However, to establish alternative labeling methods it is decisive to check detection limits for the elements in consideration. This means in concrete terms that information is required on the number of detectable element atoms per nm^2 area in the preparation. Thus, the detection limits of the ESI technique are relevant. Thus far, there are only some studies and vague indications on this parameter. Although the ESI technique is often used, no data on detection limits have been published to date.

There is thus a demand for alternative labeling methods for electron microscopy. It should be possible to readily test and assess the detectability of such a marker complex.

It is thus the object of the present invention to provide a possibility of obtaining data by means of which it is possible to evaluate the prospects of the intended experiment with the element in question and/or the marker structure in question before time-consuming cytobiological and molecular-biological experiments are carried out. Furthermore, the parameter for the detectable number of elementary atoms per unit area shall become measurable to obtain therefrom the information necessary to establish EM labeling methods.

This object is achieved by the subject matters defined in the attached claims.

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The reason why the above-mentioned preliminary tests are necessary is that thus far no accurate limiting values of detectability have been known for the ESI detection of the various chemical elements. This is *inter alia* due to the fact that preparing a suitable test sample is not a trivial matter. Such a sample must have special properties. There must be regions in which the target element is available in a clearly defined amount. It must be possible to clearly detect these regions. The target element may not occur in the remaining regions. This problem can be shown by means of the publication by Golla, U. and Kohl, H. (Micron, 28 (5), 397-406, 1997) who using uranium as an example tried to document the resolution and detectability by means of grainy precipitates.

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According to the invention a series of new plasmids having more than 1, preferably 2, 7, 14, 21 and 27, SK primer sequence elements, was produced in direct head/tail-oriented repetition on the basis of pBluescript KS(+). The annularly closed plasmid is available as a target structure which contains repetitively a short DNA sequence (SK primer sequence element). The SK primer sequence element comprises the following sequence:

5'-GATCCACTAGTTCTAGAGCG-3'.

The SK primer sequence represents a segment of 20 nucleotides which is referred to as such by the company of Stratagene in the vectors pBluescript KS(+/-) and Bluescript SK (+/-) and lies within the "multiple cloning site" (MCS). pBluescript is a plasmid vector sold by Stratagene. It is an annular DNA molecule which contains the genetic information necessary for replication in *E. coli*. The multiple cloning

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site (MCS) is decisive for cloning foreign DNA segments into this vector. According to the invention the above-mentioned SK primer sequence repetitions were incorporated into this MCS region so that the resulting plasmids of pBluescript only differ as regards the MCS, i.e. are derived from pBluescript.

A homologous sequence may be bound to this repetitive sequence by means of hybridization. In case this hybridizing sequence carries a marker complex, the marker will reach the target structure by hybridization.

The hybridizing sequence, hereinafter referred to as SK oligonucleotide or SKO, can be modified chemically at its ends to permit a covalent bond of different markers. As a result, it is possible to study any labeling strategies. A molecule can be linked to the SKO containing an element in the highest possible concentration which shall be tested for usability as a marker for ESI. Boron markers as described in German patent application 198 03 206.4, for example, are suited. Further promising markers are silicon as well as iron and manganese. The marker compound is built up in a controlled synthesis such that the number of target elementary atoms is known and the target element is present in the greatest possible amount in the center of the marker compound. It can also be linked to the SKO as a unit. In order to meet these demands, e.g. the boron marker structure is synthesized by treating it like a nucleoside unit in the oligonucleotide synthesis. Thus, the preferred linking system provides the production of a boron compound containing the necessary protective groups and linkage groups for the oligonucleotide synthesis according to the phosphoramidite method, in the course of which an oligonucleotide is built up, building block by building

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block, from the 3' end to the 5' end. In this connection, there is the possibility that for this purpose the boron complex is attached as such or in the form of a 5'-boron nucleotide(C)-3' building block to the 5' end of SKO in the last step (see in this connection also German patent application 19803206.4). It is advantageous for the marker-containing building block to contain a spacer which will cause the marker complex to stand off from the SKO so as not to impede the hybridization of the labeled SKO with the complementary plasmid regions. Aliphatic hydrocarbon chains which have lengths between C_2 and C_{10} and may possibly contain oxygen groups in the form of ether bridges (preferably a maximum of 5 bridges) are in consideration as spacers (see in this connection also German patent application 198 03 206.4). A similar procedure can be used for any other marker structures, each containing another target element.

The labeled oligonucleotides are hybridized to the DNA and are present selectively in the preparation at the sites where the annular DNA molecules are located. Depending on the repetition degree of the SK elements on the employed plasmid molecules, variable but defined amounts of target elementary atoms will thus be attached to the DNA in a very close arrangement. Therefore, densest packaging can be assumed because it was found that the distances of the marker structures on the DNA are 8 nm. This follows from a calculation of the extension of double-stranded DNA regions over the SK repetition units present in the plasmids. Since the marker structure will have a maximum diameter of 5 nm there is in fact some space available between the markers. However, this space should be maintained because the hydrate envelope of the marker compounds must be taken into account.

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The plasmid according to the invention is prepared on the carrier matrix of the sample holder for ESI in spread form. The above plasmids enable the preparation of single-stranded annular plasmid DNA molecules after infecting plasmid-containing *E. coli* cells, preferably *E. coli* JM 110, by means of what is called a helper virus. The (+) sign in the name of the original plasmid pBluescript KS (+) indicates that only the plus strand of the plasmid molecule is isolated. A single-stranded DNA sample is now available against which complementary DNA regions can readily be hybridized without the otherwise necessary fusing of the DNA duplex. In order to hybridize SK oligonucleotides (SKO) complementary with the plus strand of the plasmids, they must, of course, represent the sequence of the minus strand, i.e. 5'-CGCTCTAGAACTAGTGGATC-3'. Such an oligonucleotide can be produced by means of automatic oligonucleotide synthesis. These molecules are mixed in an aqueous solution with one of the above-mentioned single-stranded plasmid molecules. Double-stranded regions form at the sites where the SK oligonucleotides (SKO) have found the complementary partner on the single-stranded DNA, i.e. SK oligonucleotide/plasmid hybrids (hereinafter referred to as SKOPH). In order not to impede the binding of the single SKOs to the DNA, a gap of 4 nucleotides is preferably provided as a spacer between the SK oligonucleotide binding sites.

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Theses SKOPHs are preferably separated by the chromatography of unbound SKOs. This may be done by column chromatography, e.g. Amersham Pharmacia Biotech (Freiburg, Germany) offer column matrixes (e.g. sephadex or sepharose). The purified SKOPHs are then subjected to spreading. In this connection otherwise coiled DNA molecules are pretreated such that they are stretched in solution and in this state are applied onto electron-microscopic small carrier nets coated with a thin

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sheeting, made visible by treatment with heavy metals and analyzed under a transmission electron microscope (TEM). If ESI analysis shall be carried out, heavy metal treatment should be dropped, since every element occurring in high amounts and/or high concentrations in the preparation interferes with, or makes impossible, the specific detection of the target element. The DNA rings are then distributed uniformly over the surface of the TEM preparation and are separate from one another. The above-mentioned basic preconditions are thus met: the annular DNA is clearly evident, the SKOs are available in a more or less large number and are bound to the DNA, and there is (almost) nothing between the DNA regions.

In case it is not clear whether the SKOs were bound to the repetitive region, there are two possibilities for control: a) SKOs labeled at the 5' position by digoxigenin or biotin are used against which an anti-digoxigenin or an anti-biotin antibody is employed which itself is labeled with gold and can be detected by conventional TEM. The size of the gold grains may, however, not exceed a diameter of about 6 nm (otherwise the gold grains could interfere with one another); b) the repetitive target plasmid can be linearized, possibly in combination with a), by restriction endonuclease digest along with the repetitive SK region, so that following spreading the binding sites of the SKOs are readily identifiable in that they must be located at the end of a thread-like DNA molecule. Since restriction endonucleases only excise double-stranded DNA, its restriction site must first be made double-stranded by hybridization of an oligonucleotide complementary around the restriction site.

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The repetitive sequences are arranged closely one behind the other and extend over about a third of the plasmid. These repetitive sequences render the test much more significant. The advantage of the above described plasmids consists in that 1 to 27 of the marker units can be accumulated so as to modulate the number of marker elementary atoms. When it is possible to show the labeled SKOPHS in differing spreading states from fully extended to coiled in the spreading preparation, the target elementary atoms, bound particularly to coiled DNA molecules, can i) be concentrated within a very confined space, ii) become localizable due to the uniformly fibrillary ring shape of the DNA bound thereto, iii) be analyzed in defined but variable number, and iv) in an otherwise element-free environment.

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DNA segments outside the repetition regions to which no marker can bind serve as a negative control for the ESI elemental detection. Such a negative control is necessary because the specificity of a calculated target element distribution could be doubted if there was no comparative region without target element and correspondingly without calculated element signal. Since the test represents a molecular-biological system, the marker is assessed in its physico-chemical environment. This also means that the test is very close to a medical/biological application, in particular the *in situ* hybridization.

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It is the objective of this test method to obtain reliable data on the minimum number of target element atoms per unit area necessary for ESI detection. At the same time, data are obtained on the individual detectability of the marker structure because due to the repetition thereof it is also possible to average weak element-specific signals, in particular in DNA molecules available in the electron-

microscopic preparation in a fully stretched manner. Thus, it can already be determined prior to a technically complicated use of a marker structure in medicine or biology whether optionally the number of/and the concentration of the target elementary atoms must still be increased in the marker structure. As experience shows, all of the plasmid states from stretched to considerably coiled are found in spreading preparations, in particular when the spreading process did not proceed in optimum fashion. This usually undesired case is of advantage in connection with the explained determination of elemental detection limit.

Many variations of spreading methods are found in the literature (regarding a summary see: Electron Microscopy in Molecular Biology; a practical approach, Sommerville, J. and Scheer, U. (eds.), IRL Press, 1987).

The threshold values for the element-specific detection can be determined by the standard methods of elemental detection using ESI. For this, there is presently no other method. It can thus be imagined that this method is also of interest for the scientists who do not have in mind a biologically/medical use but are interested in the detection limits of any chemical elements other than those mentioned above. The precondition is that the target element is already present in the marker structure linked to the oligonucleotide in the highest possible concentration and in the greatest possible amount.

The use for ESI has been specified above. In addition, applications of parts of the test system are also possible which go beyond the use in electron microscopy. Two further sample applications are mentioned here briefly and specified below: 1) the SK primer repetition cassette can generally be

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utilized for the efficient and localized DNA labeling by hybridizing labeled oligonucleotides; 2) for studying the mechanisms of deleting direct repetitions in DNA the below described plasmids form the model substrate.

In addition to the application in the field of electron microscopy, said repetition regions enable, as stated above, the possibility of also rendering DNA quite generally detectable after the hybridization by means of the SKO-linked markers which are still below the detection limit as single molecules but can be identified in repetitive arrangement. For this purpose, the repetition regions can also be recloned into the desired DNA molecules via SAC I/Kpn I-compatible ends. For example, the route of DNA can then be tracked by such a method after introduction into a cell (transfection). Here, the uses of both optical and electron microscopes are in consideration.

The chemical modifiability of the hybridizing sequence permits different uses of the test for differently configured marker units. Since the test represents a molecular-biological system, the marker is assessed in its physico-chemical environment. The individual detectability of the marker is analyzed. The intensity of weak signals can be determined precisely by averaging.

The following statements made on the production of the plasmids containing different repetitions show that in principle the repetition steps (pBluescript KS (+)) 2x, 3x, 4x, 5x, 6x, 7x, 14x, 21x and 27x SK are available for the experiments for the actual elemental detection for ESI. Since the analysis of differences as to the detectability of the target element using ESI will be especially convincing when the number of analyzed elementary atoms varies

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considerably (see in this connection the below statements), the repetition degrees 2x, 7x, 21x and 27x SK are of special interest.

As mentioned above, the present invention is based on the fact that a test preparation contains areas in which the target element is available in a clearly defined amount and can be detected unambiguously. The target element may not occur in the remaining areas.

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The plasmid construction is stabilized by introduction into a *dam*⁻/*dcm*⁻ strain (preferably *E. coli* JM 110). JM110 is *dam*⁻/*dcm*⁻ and contains no other striking genotypic markers which would clearly distinguish this strain from the other ones used, so that they can be employed as well. The repetitive plasmids according to the invention are introduced into the *dam*⁻/*dcm*⁻ strain according to standard methods (cf. Sambrook, J., Fritsch, E.F. and Maniatis, T.: Molecular cloning; A laboratory manual; Second edition, Cold Spring Harbor Laboratory Press (1989)). Surprisingly, a deletion of the directly repetitive elements is thus avoided during bacterial replication. It is actually known that direct repeats or inverted repeats are lost during the replication in *E. coli*. *dam*/*dcm* strains are documented in the literature (cf. Marinus et al., J. Bacteriol. 114 (3), 1143-1150 (1973)); however, stabilization of directly repetitive sequences, resulting therefrom, has never been described.

The repetition degree could even be increased to 27x in *E. coli* JM110. Furthermore, the combination of *E. coli* JM110/pB1 KS (+) 27xSK is for the first time a system in which a direct repetition sequence otherwise unstable in *E. coli* can be replicated. Bacterial geneticists get the

possibility of analyzing the underlying mechanisms of this type of deletions in bacteria and characterize the involved components. The question of stabilizing repetitions of such a type in *E. coli* is of interest e.g. for cloning specialists who try to obtain human DNA segments in their original state even if they had been replicated in *E. coli* (see e.g. human genome project). The background is that human DNA segments also contain short directly repetitive segments which like the above-described SK primer sequence repetition can show relatively poor stabilizing characteristics.

The plasmids according to the invention can be combined into test kits for use in electron microscopy. A test kit contains e.g. the following materials: 1) competent *E. coli* JM110 bacterial cells for replicating the repetitive plasmids; 2) the single-stranded plasmids 1x or 2x, 7x, 14x, 21x and 27x SK for the differential analysis of marker structures for the electron microscope; 3) electron-microscopic small carrier nets which are already coated for spreading; 4) SK oligonucleotides labeled at their 5' end by biotinylation or digoxigenation and serving for optimizing hybridization and spreading by proving by means of a gold-linked anti-biotin or anti-digoxigenin antibody that the repetitive arrangement is actually given on the plasmid; 5) instructions describing the individual processing steps. If there is an interest in applications other than the ESI-dependent ones, the test kit can be modified for those interested.

The plasmids having 2, 7, 14, 21 and 27 SK primer sequence elements were deposited as *E. coli* cultures with DSMZ (*Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*) [German-type collection of microorganisms and cell

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cultures], Mascheroder Weg 1, Braunschweig, Germany, under accession numbers DSM 12600, DSM 12601, DSM 12602, DSM 12603, and DSM 12604 on December 22, 1998:

pBI KS(+) 2xSK	DSM 12600
pBI KS(+) 7xSK	DSM 12601
pBI KS(+) 14xSK	DSM 12602
pBI KS(+) 21xSK	DSM 12603
pBI KS(+) 27xSK	DSM 12604

The following figures explain the invention in more detail.

Figure 1: Outline of the production of pBI KS(+) 2x SK.

The kind of presentation given here is continued in the following illustrations of this kind. The multiple cloning site (MCS) is shown as a dark-gray block, and the SK primer sequence included therein is light gray. The restriction sites are marked by a finely broken line. The detailed sequence is indicated by the segments important for cloning.

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- Diagram of pBI KS(+). pBI KS(+) was digested with the restriction enzymes Kpn I and BamH I for subsequent cloning. The restriction sites are marked by a finely broken line. The MS fragment therebetween falls out.
 - Diagram of pBI KS(+) digested with BamH I and Kpn I and the SK-PH I fragment which should result in pBI KS(+) 2x SK by ligation with pBI KS(+). Part of MCS was excised by digest with Kpn I and BamH I (see also a), and the fragment SK-PH I was inserted in return. Using SK-PH I the previously present Bam H I restriction site was masked by means of modification of a base pair (bold letters) and a new BamH I restriction site was introduced at the same time. Due to the different

restriction sites (Kpn I/BamH I) the fragment can only be cloned in a possible orientation. The restriction site Pvu I served as a control restriction site for the successful incorporation of the insert SK-PH I (no further data shown in this connection).

- c) Diagram of pBl KS(+) 2x SK. pBl KS(+) 2x SK was formed by ligation of SK-PH I with the BamH I/Kpn I digested pBl KS(+) (cf. b). The modified BamH I restriction site marked by an asterisk could no longer be excised by BamH I. In order to simplify the following text, the region marked in the illustration (SK primer + non-hybridizing sequence) is marked by a black arrow. This leads to the schematic plan for pBl KS(+) 2x SK as shown under item d).
- d) Simplified presentation of pBl KS(+) 2x SK. The full black line stands for MCS, the broken line stands for the remaining vector pBl KS(+). The black arrows show the 5' → 3' direction of the cloned SK primers plus 4 bp sequence not to be hybridized (cf. item c).

Figure 2: simplified diagram of pBl KS(+) 7x SK.

The pBl vector is marked by a broken black line; seven SK primer sequences are now contained in its Kpn I/Sac I-oriented MCS. The SK-PH II fragment (dashed arrow on the top and sequence "SK-PH II" emphasized by lines at the bottom) inserted the seventh SK primer and the additional Eag I restriction site in the vector. The important sequences are emphasized in detail. The SK primer sequence is light gray, the rest of MCS and the 4 base spacers are dark gray. The restriction sites are marked in the sequence by a finely broken black line.

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Figure 3: Diagram of cloning a pBl2x block plasmid

- a) Insertion of a 7x SK block in the Not I-opened pBl 1x block DNA. The characterization of the individual components is identical with those of figure 1 or figure 2. The clone pBl 1x block was linearized with the Not I enzyme and ligated with the PCR fragment subsequently cut by Eag I beforehand. In order to simplify the following text, the seven SK fragments are combined into a gray arrow.
- b) Presentation of the transitions between individual blocks. The marking of the components can be compared with that in figure 1.a-d. By ligation of the 7x SK block (gray arrow) in the proper orientation, the Not I restriction site which opened the pBl 1x block beforehand was masked by the 5' end of the newly added 7x SK block (bold letters) and could no longer be excised by Not I. The 3' end of the fragment completes the Not I restriction site towards the vector. As a result, it is possible in the next cloning run to again linearize the pBl 2x block with Not I without losing the 14 SK primer. In contrast to the BamH I cleavage site between the individual SK primers in the block (BamH I*), the BamH I restriction site at the 5' end of a 7x block is maintained (BamH I) and can subsequently be used as an orientation control.

Figure 4: Sequencing result of the plasmid construct containing 27 SK primer elements

Black bars mark the SK primer sequence regions in the repetitive region sequenced from both sides. Sequences ATCT or GCCG which have a length of 4 base pairs are located between these SK primer sequence regions for reasons of cloning technique.

Figure 5: Diagram of the labeling experiment

The following example explains the invention in more detail.

Example

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The below described methods of producing the plasmids containing the repetitions are described in Sambrook, J., Fritsch, E.F. and Maniatis, T. (Molecular cloning; a laboratory manual; second edition; Cold Spring Harbor Laboratory Press, 1989) and in Current Protocols in Molecular Biology (John Wiley and Sons, 1994-1998), the below techniques, such as DNA replication, restriction endonuclease digest, ligation, agarose gel electrophoresis, and PCR being sufficiently known to, and mastered by, a person skilled in the art.

The SK primer element of Bluescript was selected for the repetition (Stratagene company, Heidelberg, Germany) because it does not contain any self-complementary or homooligomeric regions, with a G/C content of 50 % it lies within the average region of natural DNA and is suited for the almost full production of directly repetitive regions as regards the cloning technique. Furthermore, it is advantageous that this region hybridizes reliably and stably with a complementary sequencing primer (identical with the SK primer described herein) since it has been designed by Stratagene (Heidelberg) as a sequencing primer binding site.

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A short oligonucleotide fragment is required for the construction of SK primer sequence elements in repetitive succession. It contains the SK primer sequence and restriction sites for carrying out cloning. For this purpose oligonucleotides complementary to one another were

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synthesized. These ss-DNA fragments were converted by hybridization into clonable ds fragments by juxtaposing the two complementary oligonucleotides in equimolar fashion in 10 mM Tris buffer. Accomplished cloning is a control for the success. The resulting fragments were referred to as SK-PH I (SK primer hybrid I; fragment which was used for the SK primer replication of 2 - 6 SK primer sequences; see Ill. 1) and SK-PH II (SK primer hybrid II; fragment which introduced the seventh SK primer and the Eag I restriction site; see Ill. 2).

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For the production of the plasmid pBl KS(+) (pBluescript KS(+)) with two SK primers (pBl KS(+) 2x SK), the pBl KS(+) had to be opened using BamH I and Kpn I, part of the multiple cloning site MCS having been removed (Ill. 1a). The complete double digest was identified on a 2 % agarose gel, followed by ethanol precipitation. The insert SK-PH 1 (Ill. 1b) was added to the opened vector for ligation in a tenfold excess (see Ill. 1b). This high excess could be justified since the 5' ends of the fragment were not phosphorylated, i.e. oligomers of the inserts could not form. The transformation in *E. coli*, e.g. XL1-Blue, was carried out using this ligation batch. Of the raised colonies the plasmid DNA was isolated by mini-preparation from three clones for cloning control. The resulting clones are indicated below as pBl KS(+) 2x SK (Ill. 1c).

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The further cloning of plasmids with up to seven SK elements contained in equal orientation was time-consuming, since one clone from the last cloning run served in each case as a basis for the next cloning step. Correspondingly, the midi-prep-DNA of the select pBl 2x SK clone was again double-digested by BamH I/Kpn I and admixed with SK-PH I, ligated and transformed in *E. coli* XL1-Blue. Contrary to the

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strategy used for cloning pBl 2x SK, attention had then to be paid especially to an efficient double digest using BamH I and Kpn I. As shown in Ill. 1c, the restriction sites into which another SK-PH I fragment should be integrated, were only six base pairs apart from one another. Such a small distance between two restriction sites does not permit the simultaneous restriction of both restriction sites. Correspondingly, the restriction had to be carried out successively using the two enzymes. Cloning up to the plasmid pBl KS(+) 6x SK was carried out in this way.

Having cloned the seventh SK primer sequence, the repetitive elements were replicated block-wise. This could only function with a restriction site separating the region with seven SK primers as a unit from the vector. This was enabled by ligation of the SK-PH II (Ill. 2) in pBl 6x SK. Along with the seventh SK element, SK-PH II introduced the new restriction site Eag I into the vector. The seven SK primers were then confined by two Eag I restriction sites (Ill. 2) because the starting vector pBl KS(+) already had such a restriction site in the MCS.

In order to accelerate the further cloning steps, the block-wise replication of the SK elements was made by means of polymerase chain reaction (PCR). The plasmid preparation from XL1-Blue was taken as a template DNA for the amplification of the fragment with seven repetitive elements. It was derived directly from the original colony (pBl 7x SK). In a first optimization of the PCR it should be analyzed which primer pair amplified the target fragment having the best quality and quantity. The primers M13, M13 reverse, T3 and T7 (M13: TGTAACACGACGGCCAGT; M13 reverse: CAGGAAACAGCTATGACC; T3: AATTAACCCTCACTAAAGGG; T7: TAATACGACTCACTATAGGG) were tested in various combinations.

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All of these primers had their binding sites outside the MCS, either close to the β -galactosidase starting point or close to the T7 transcription starting point in pBluescript KS (+). PCR took place under standard conditions. The various batches contained the matching primers in the various combinations possible: M13/M13 reverse, T7/T3, T7/M13 reverse and T3/M13. All of the four primers were combined without the template in the negative control. Since the PCR batch supplied the best results with T3/T7, this primer pair was used for the PCR.

In order to be able to ligate the insert fragment obtained by means of PCR into the NotI-opened vector, it had to have sticky ends compatible with Not I. For this purpose, the PCR fragment which contained the seven SK primers had to be subsequently cut at the edges. The restriction enzyme Eag I shortened the 246 bp long PCR fragment whose edges closed the sequences of primers T3/T7 by 47 bp and by 51 bp on the other side. This difference with respect to the control could still be made visible using a 2.2 % gel. pBl KS(+) 7x SK changed into pBl KS(+) 14x SK in only one step using the PCR-amplified 7x SK fragment whose edges became compatible with Not I by Eag I digest. See Ill. 3 for an outline of the cloning manner. The digested fragment was purified prior to ligation by the PCR purification kit (Qiagen company). This should serve for removing the primers not consumed in the PCR reaction and the fragments resulting from the digest.

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As compared to the first cloning steps which resulted in pBl KS(+) 7x SK, the vector was not opened by two different enzymes (Kpn I/BamH I; see Ill. 1) but linearized by Not I. Therefore, an accumulation of religations had to be expected. In this cloning, a religation could not be counteracted by an insert concentration increased many times

over (7x SK fragment), since the DNA blocks were phosphorylated at their 5' ends and uncontrollable oligomerizations of the insert DNA had to be expected. Therefore, the religations were reduced, or even suppressed, by dephosphorylating the vector. The DNA referred to as pBlKS(+)7xSK thus far is designated pBl 1xblock below.

The pBl 1x block opened by Not I was ligated with the purified PCR fragment which also contained seven SK primers. This was enabled by the single Not I restriction site located at the edge of the repetitive elements, by which the pBl 1x block was linearized. As mentioned above, the PCR fragment was subsequently cut with the Eag I enzyme compatible with Not I and ligated directly to the seven SK primers of the vector (pBI 2x block).

Since pBl 7x SK proved to be stable in the JM110 host strain, the ligation batch of the plasmid with 14 SK elements was also transformed into this strain. The transformation of pBl KS(+) 14x SK in JM110 yielded 118 transformants. This corresponded to a transformation rate of 1.7×10^3 cfu (colony forming units) / μg DNA.

Step-wise replication was used, in this case with the aim of building up with the 7x SK blocks a plasmid having 28 repetitive SK primer sequences. For this, the pBl 2x block was linearized by Not I as shown analogously in Ill. 3. The full digest was examined on a 1 % agarose gel. The 5' ends of this Not I-opened plasmid were dephosphorylated and ligated with the 7x SK block. Seven colonies resulted from this transformation.

Control digest with BamH I of several candidate clones showed that a complete 7x SK block had additionally been

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inserted. One of the clones was replicated for a midi preparation and the DNA was prepared. The sequence analysis from this midi preparation identified the complete and correct sequence of 21 SK primers including the functioning restriction sites which were required for the next cloning run. The gel analyses were confirmed in this connection. This clone is referred to as pBl 3x block below. It served as a precursor for the next insertion run.

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In order to obtain a plasmid having 28 repetitive SK elements, the block-wise replication of the 7x SK block was continued. The pBl 3x block was used as a starting plasmid of this cloning. This cloning was treated like the two preceding ones. The pBl 3x block was linearized using Not I, checked for full digest in a 1 % agarose gel and then dephosphorylated. The dephosphorylated vector was used together with the PCR-amplified and subsequently cut 7x SK block in a ligation batch. The control ligation for evaluating the dephosphorylation yielded 2 clones. 59 clones formed in the transformation of the ligation with insert, 16 colonies of which were selected for a mini preparation. Separation with an agarose gel after Sac I/Kpn I digest was made as usual on a 2.2 % gel matrix.

09044397 030102
201002 26241660

A total of 5 clones had prolonged insert regions. Control digests with BamH I and triple digests with Sac I/Kpn I/BamH I showed fragment patterns indicating that no complete 7x SK block might have joined. A BamHI site must have been deleted in the newly joined block during the cloning instead.

One of the five equal clones was chosen and a sequence analysis was made using its midi-prepared DNA. Sequencing confirmed the result that the newly joined BamH I restriction site was deleted. The complete SK primer with

Sub
B22

intact BamHI restriction site of the last joined 7x SK block lacked. The result was thus a pBl KS(+) plasmid having 27x SK primers. The sequence of this clone is shown in figure 4.

B22
correct

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